TITLE OF THE INVENTION

QUANTITATION OF RNA

FIELD OF INVENTION

The present invention relates to the quantitation of RNA present in tissue, thereby permitting analysis, for example, of rare transcript expression in cells.

BACKGROUND OF THE INVENTION

It is known in the art how to amplify DNA by polymerase chain reaction (PCR). It has also been shown that mRNA can be transcribed into DNA templates and then amplified by PCR in a method known as RT-PCR. The limitation of existing RT-PCR methods is that some very rare transcripts (mRNAs) are unable to be amplified in the RT and subsequent PCR portion of the existing methods. It would be a great benefit in the analysis of subtle changes in the expression levels of certain genes, for example, cytokine genes in non-lymphode tissue, to be able to not only to detect but quantify the levels of these transcripts after treatment of the host.

Previously RT-PCR was performed in two distinct steps. This involved the reverse transcriptase step in which mRNA was transcribed into DNA mediated by the enzyme reverse transcriptase. This enzyme is heat labile so the temperature at which cDNA synthesis was effected had to be within a limited temperature range. The next step involved destroying the activity of the RT by heat-inactivation and then adding the DNA polymerase (PCR step) to initiate the amplification step.

By using a recombinant *Thermus thermophilus* (rTth) enzyme that possesses both RT activity and DNA

polymerase activity, in the presence of manganese one can reduce and simplify the RT-PCR method to a one-enzyme procedure. Also, using a heat-stable enzyme such as, rTth, one can increase the annealing temperature during the RT stage to reduce the non-specific cDNA generated through non-specific priming, ensuring only the target RNA sequence is transcribed into DNA. This then greatly increases the ability to amplify the target DNA in the next step, the PCR reaction.

Methods of isolating RNA are known, for example Graham et al., (Ref. 1 - various references are referred to in parenthesis to more fully describe the state of the art to which this invention pertains. Full bibliographic information for each citation is found at the end of the specification, immediately preceding the claims. The disclosure of these references are hereby incorporated by reference into the present disclosure). The yields from these methods tend to be very low. When the desired target is a very rare message, these types of isolation procedures do not yield enough total RNA to detect the very rare message. Therefore, it is desirable to improve the yield of the total RNA from various tissue samples so the rare messages are represented. In addition, if the yields of RNA from certain tissues, for example, lungs, are increased, this reduces the need to pool several samples from different animals, thereby reducing the biological diversity. This allows the researcher to define what is happening in a specific animal after treatment.

To investigate the expression levels of rare messages, it is important to extract from the tissue of

interest enough intact total message to enable amplification and quantitation of these rare messages.

SUMMARY OF THE INVENTION

The present invention relates to the determination of RNA production in cells. By modifying existing procedures, the invention permits an accurate quantitation of small quantities of RNA, representative of rare transcripts in tissues or cells, in particular the quantitation of cytokine RNA in mouse lung and spleen tissue.

While other methods of quantitative PT-PCR compare the final PCR product to the level of expression of an endogenous gene or an internal cDNA standard (Refs. 2, 3, 4), the inventors have designed, in one embodiment of the present invention, an RNA standard that binds the same specific primers and probe as the target sequence utilizing a fluorescent tag. This helps equalize the efficiency of the large RT-PCR reaction for the standard RNA and target mRNA in a sample, which are sensitive both to changes in template length and secondary structure. If these reactions are not equivalent, true quantitation cannot be determined.

In accordance with one aspect of the present invention, there is provided a method of determining the quantity of a target RNA in a tissue sample, which comprises isolating the tissue sample from a host; isolating total RNA from the tissue sample; subjecting the isolated RNA to a reverse transcriptase reaction followed by a DNA polymerase amplification reaction (PCR) using primers corresponding to transcribed sequences of the target RNA; binding a labelled sequence corresponding to an internal sequence complementary to one of the strands of the PCR product

of the target RNA; and determining the amount of labelled sequence bound to amplified transcribed target RNA.

In another aspect of the present invention, there is provided a method of quantifying more accurately a target RNA in a tissue sample, which comprises isolating the tissue sample from a host; isolating total RNA from the tissue sample; subjecting the total RNA to a reverse transcriptase reaction followed by a DNA polymerase amplification reaction using primers corresponding to transcribed sequences of the target RNA; binding a labelled sequence corresponding to an internal sequence of the amplified product; and determining the amount of bound labelled sequence by the generation of a detectable label. The detectable label may be fluorescence.

The comparison of the determined amount of bound labelled sequence to an RNA standard may be effected by regression analysis using the plot.

An RNA standard of the number of copies of target RNA may be established. The determined amount of bound labelled sequence in the sample then may be compared to the RNA standard as a measure of the number of copies of target RNA in the tissue sample.

The RNA standard employed in this aspect of the present invention may be established by synthesizing an RNA molecule corresponding to the target RNA; quantifying the synthetic RNA molecule; effecting serial dilution of said synthetic RNA molecule to provide a plurality of samples of known starting copy number; subjecting the synthetic RNA molecule in each sample to a reverse transcriptase reaction followed by a DNA polymerase amplification reaction using primers

corresponding to transcribed sequences of the synthetic RNA molecule and corresponding in sequence to those employed during DNA polymerase amplification of the transcribed sequence of the target RNA; binding a labelled sequence corresponding to an internal transcribed sequence of the synthetic RNA molecule to amplified transcribed synthetic RNA molecule in each sample, the labelled internal sequence being the same as that used to bind to amplified transcribed target RNA; determining for each sample the quantity of labelled sequence bound to amplified transcribed target RNA; and plotting the individual determinations of the quantity of labelled sequence in each sample against the log of the known starting copy number for each of the samples to provide a plot.

The detection step may be effected in one of two ways. In one such alternative, the detection step is effected by hybridizing the labelled sequence to the amplified product following the amplification step, and including the step of separating bound labelled sequence from unbound labelled sequence prior to the determination step. The label used in this procedure may be radioactive.

In the other alternative procedure, the detection step is effected by effecting the DNA polymerase amplification in the presence of the labelled sequence and the determination of the amount of bound labelled sequence is effected by detecting the generation of a detectable label. The detectable label may be fluorescence.

Preferably, the reverse transcriptase and DNA polymerase amplification reactions (RT-PCR) are both carried out using a single enzyme reaction, preferably

using *Thermus Thermophilus* enzyme, which may be found recombinantly.

The detection of the fluorescent signal can be achieved through the use of a real-time detection system, such as described in ref 3.

The primers used in the DNA polymerase reaction may correspond to the 5' and 3' ends of an internal transcribed sequence of the target RNA.

BRIEF DESCRIPTION OF DRAWINGS

Figure 1 shows a construction scheme for the assembly of plasmid pLITMUS-IFN-γ;

Figure 2 shows the nucleotide sequence for pLITMUS-IFN-γ (SEQ ID NO.9);

Figure 3 shows the nucleotide sequence for the IFN-γ RNA standard (SEQ ID NO. 10);

Figure 4 shows graphically the RNA standard used to determine the quantity of RNA in a sample;

Figure 5 shows optimization of the PCR cycle for single-tube RT-PCR for IL5(a), γ-IFN(b) and IL4(c) a total RNA from mouse lungs; and

Figure 6 shows the quantity of γ-IFN determined in lung samples taken from mice immunized with PBS (#529, 550, 551), live RNA (#549, 509) and formalin-inactivated RSV (#569).

GENERAL DESCRIPTION OF THE INVENTION

RNA isolation may be effected in any convenient manner as long as there is no contamination of genomic DNA which would give a false signal. In one embodiment of the invention, RNA isolation is effected by homogenization of the sample tissue in the presence of TRIzol Reagent (GIBCO/BRL), a mono-phasic solution of phenol and guanidine isothiocyanate. During sample

homogenization or lysis, TRIzol Reagent maintains the integrity of the RNA, while disrupting cells and dissolving cell components. It is important to eliminate any trace organic solvent on the homogenization probe after washing to prevent the DNA migrating from the interphase layer and mixing with RNA in the aqueous phase.

Addition of chloroform to the homogenized sample followed by centrifugation, separates the solution into an organic phase, an interphase layer containing DNA and protein contaminants, and an aqueous phase, where the RNA is exclusively located.

After separation of the aqueous phase, the RNA may be separated therefrom by precipitation through mixing with isopropyl alcohol or other suitable alcohol and centrifugation. The supernatant is removed and the RNA pellet is washed once with ethanol followed by air drying the pellet.

This conventional RNA isolation procedure is improved by washing the homogenization probe between samples in the following manner. The probe first is washed with sterile water then ethanol and then twice with sterile water using wash tubes. The probe is wiped off with a sterile wipe both after the initial and final sterile water wash before proceeding with the homogenizing of the first tissue samples. This washing operation removes residual ethanol and prevents sample carryover which may lead to DNA contamination of the final product.

Reverse transcriptase PCR amplification of the RNA is next carried out, preferably, via a single enzyme reaction using rTth DNA polymerase and appropriate primers and probe. The use of such recombinant enzyme

permits higher temperatures for RT incubation, which leads to the generation of more specific DNA.

The gene segments amplified from the transcribed RNA depend on the target RNA under investigation. For example, for murine cytokines, the applicants have identified the following gene segments:

Murine 1L-4: na 249 to 363 (SEQ ID Nos. 1, 2)

Murine 1L-5: na 336 to 402 (SEQ ID Nos. 3, 4)

Murine 1L-10: na 401 to 495 (SEQ ID Nos. 5, 6).

Murine IFN-γ: na 404 to 507 (SEQ ID Nos. 7, 8)

Determination of the quantity of RNA in the specific tissue sample is possible using any convenient label or tag, such as the PE ABI 7700 fluorescence detection system and Taqman chemistry. The use of a fluorescence detection system permits quantitation of RNA in tissue down to a level of 100 molecules.

The determination of the quantity of RNA in the sample is effected, in one embodiment, using an RNA standard, as described above. By first developing a plot of the relationship of the quantity of label to the number of copies of a target RNA in a sample, the unknown quantity of RNA in a given sample can be determined by reading off the value from the plot. The same primers and probe are used both in the development of the standard and in a sample determination to ensure an accurate measure of target RNA in the sample.

The procedure of the present invention enables an improved yield and purity of RNA to be obtained in comparison to published data, such as in the Graham et al reference referred to above. Rather than tissue from two mice being necessary to obtain sufficient RNA

for amplification, individual mice can be analyzed and at least over 200 assays are possible for each RNA sample.

Whole tissue is analyzed as opposed to RNA extracted from selected cells, as described in Waris et al. above.

SEQUENCES

Mouse IL-4 sequence (sense strand (SEQ ID No: 1) with anti-sense (SEQ ID No: 2) below):

CGTCCTCACA GCAACGAAGA ACACCACAGA GAGTGAGCTC GTCTGTAGGG CTTCCAAGGT 60
GCTTCGCATA TTTTATTTAA AACATGGGAA AACTCCATGC TTGAAGAAGA ACTCT 115
- SEQ ID No. 1

GCAGGAGTGT CGTTGCTTCT TGTGGTGTCT CTCACTCGAG CAGACATCCC GAAGGTTCCA 60
CGAAGCGTAT AAAATAAATT TTGTACCCTT TTGAGGTACG AACTTCTTCT TGAGA 115
- SEQ ID NO. 2

Mouse 1L-5 sequence (sense (SEQ ID No: 3) with anti-sense (SEQ ID No: 4) below):

ACCGCCAAAA AGAGAAGTGT GGCGAGGAGA GACGGAGGAC GAGGCAGTTC CTGGATTACC 60
TGCAAGA - SEQ ID No: 3 67

TGGCGGTTTT TCTCTTCACA CCGCTCCTCT CTGCCTCCTG CTCCGTCAAG GACCTAATGG 60
ACGTTCT - SEQ ID No: 4 67

Mouse IL-10 sequence (sense strand (SEQ ID No: 5) with anti-sense (SEQ ID No: 6) below).

TGAATTCCCT GGGTGAGAAG CTGAAGACCC TCAGGATGCG GCTGAGGCGC TGTCATCGAT 60
TTCTCCCCTG TGAAAATAAG AGCAAGGCAG TGGAG - SEQ ID No. : 5 95

ACTTAAGGGA CCCACTCTTC GACTTCTGGG AGTCCTACGC CGACTCCGCG ACAGTAGCTA 60
AAGAGGGGAC ACTTTTATTC TCGTTCCGTC ACCTC - SEQ ID No. : 6 95

Mouse interferon-gamma mRNA sequence (sense strand (SEQ ID No: 7) and anti-sense (SEQ ID No: 8) below):

CATTCATGAG TATTGCCAAG TTTGAGGTCA ACAACCCACA GGTCCAGCGC CAAGCATTCA 60
ATGAGCTCAT CCGAGTGGTC CACCAGCTGT TGCCGGAATC CAGC - SEQ ID NO. : 7 104

GTAAGTACTC ATAACGGTT CAAACTCCAG TTGTTGGGTG TCCAGGTCGC GGTTCGTAAG 59
TTACTCGAGT AGGCTCACCA GGTGGTCGAC AACGGCCTTA GGTCG - SEQ ID No. : 8 104

EXAMPLES

The above disclosure generally describes the present invention. A more complete understanding can be obtained by reference to the following specific Examples. These Examples are described solely for the purposes of illustration and are not intended to limit the scope of the invention. Changes in form and substitution of equivalents are contemplated as circumstances may suggest or render expedient. Although specific terms have been employed herein, such terms are intended in a descriptive sense and not for the purposes of limitations.

Example 1:

This Example describes a general procedure of RNA isolation.

100 mM 2-mercaptoethanol in TRIzol® reagent was prepared. Frozen tissue was homogenized in 1.0 ml 2-ME/TRIzol® per 100 mg tissue with Polytron generator at setting 6 for 50 seconds and 40 seconds, with a 10 second rest in between. Washes of generator probe were performed before beginning the procedure and between each sample. These consisted of homogenization of 30 mL volumes of sterile solutions for 30 seconds at setting 6 with water, 70% ethanol, and two further washes with water. Wiping of the generator probe after the initial and final water wash was performed with a sterile wipe. Additionally, a brief pulse of probe while enclosed

within a wipe was performed at the end of the washes to remove any trapped liquid. After a ten minute incubation at room temperature, the homogenized sample was transferred to a 1.5 mL tube containing 200 µL chloroform and shaken for 10 seconds. Following a 3 minute incubation at room temperature, the phases were separated by centrifugation at 12,000 x g for 15 minutes at 4°C. 400 µL of the aqueous (upper) phase was transferred to a 1.5 mL tube containing 330 µL isopropyl alcohol and vortexed for 10 seconds. After 10 minutes incubation at room temperature, the RNA was pelleted by centrifugation as before. After removal of the supernatant, the pellet was washed with 1.0 mL 75% ethanol. The tube was vortexed for 10 seconds and centrifuged at 7,500 x g for 5 minutes at 4°C. After removal of the supernatant, the RNA pellet was dried for 5 minutes at room temperature. The RNA was dissolved in DEPC-treated water and quantitated by absorbance at 260 nm in a uv spectrophotometer.

Example 2:

This Example describes a general procedure single-tube RT-PCR.

cDNA was synthesized from 100 ng of RNA utilizing Mn^{2+}-dependent reverse transcriptase activity of recombinant DNA polymerase rTth from *Thermus thermophilus* and sequence-specific primers. PCR amplification followed in the same tube, also utilizing rTth polymerase. Each RT-PCR was performed in a 20 µL-final volume, with final concentrations of 50 mM Bicine 115 mM potassium acetate, 8% (w/v) glycerol, 0.3 mM dNTPs, 0.4 mM primers, 4 U of rTth polymerase, 8 U RNAsin RNAse inhibitor, and 2.5 mM $Mn(OAc)_2$ at pH 8.2.

Reverse transcription was performed between 55°C and 65°C for 60 minutes, the actual temperature being optimized for each gene target. The first denaturation was performed at 94°C for 1 minute and the amplification cycles were 94°C for 15 seconds, 55°C for 30 seconds, and 70°C for 30 seconds. The cycle number for each target was optimized to ensure the increase in PCR product was in the exponential growth phase. The results of optimization experiments for IL5(a), γ-IFN(b) or IL4(c) using 25, 30, 35 or 40 cycles are shown in Figure 5.

The RT-PCR products were allowed to hybridize in solution to a ^{32}P-end-labelled oligonucleotide probe, specific for an internal region of the target sequence. Hybridized fragments were separated from unbound probe using 12.5% poly-acrylamide gel electrophoresis. Dried gels were exposed to X-ray film or scanned for radioactivity on a β-scanner.

Example 3:

This Example describes a specific quantitation of γ-interferon expression levels using RT-PCR.

cDNA was synthesized from 100 ng of RNA utilizing Mn^{2+}-dependent reverse transcriptase activity of recombinant DNA polymerase rTth from *Thermus thermophilus* and sequence-specific primers designed by Primer Express® software. PCR amplification followed in the same tube in the presence of a TaqMan® probe specific for an internal sequence of the target gene, also utilizing rTth polymerase. Each RT-PCR was performed in a 25 µL-final volume, with final concentrations of 50 mM Bicine, 115 mM potassium acetate, 0.01 mM EDTA, 60 nM passive reference 1(ROX

dye), 8% (w/v) glycerol, 0.3 mM dNTPs, 0.1 to 0.4 mM primers, 5 U of rTth polymerase, 10 U RNAsin RNAse inhibitor, and 1.0 to 5.0 mM $Mn(OAc)_2$ at pH 8.2 according to manufactures instructions in the TaqMan EZ RT-PCR core reagents (Perkin-Elmer). Reverse transcription was performed at 60°C for 30 minutes. The first denaturation was performed at 95°C for 3 minutes and 40 amplification cycles were as follows: 95°C for 15 seconds, 60°C for 30 seconds, and 70°C for 30 seconds. The RT-PCR products were measured by increases in fluorescent signal above a threshold level (threshold cycle, or C_T). C_T values vary inversely with the amount of nucleic acid in the original sample. Regression analysis was performed using the standard curve of Ct values against log copy number (Figure 4; Example 5), to determine the copy number in each sample based on its experimental Ct value.

Copy number values obtained for mice immunized with PBS (#529, 550, 551), live RSV (#549, 509) or formalin inactivated RSV (#569) are shown in Table I below and plotted graphically in Figure 6.

Example 4:

This Example describes the construction of plasmid pLITMUS-IFN-γ as outlined in Figure 1.

The pLITMUS-IFN-γ plasmid was created by digesting plasmid pMPI3mIF (obtained from Virogenetics Corp.) with the restriction enzymes *Hind*III and *Eco*RI isolating the 624 bp fragment and ligating this fragment into the LITMUS-28 plasmid (New England Biolabs, Mississauga, Ontario, Canada) that had also been cut with *Eco*RI and *Hind*III. The sequence of plasmid pLITMUS-IFN-γ is shown in Figure 2. Plasmid

pMPI3mIF contained a 498 bp cDNA fragment of the IFN-γ mRNA. Although cDNA fragment represents less than half of the IFN-γ mRNA, it includes all of the IFN-γ coding region.

Example 5:

This Example describes the preparation of *Spe*I linearized pLITMUS-IFN-γ required for the generation of the IFN-γ RNA standard in an *in vitro* transcription reaction and the preparation of the IFN-γ RNA standard.

20 μg of pLITMUS-IFN-γ DNA was cut with *Spe*I in a reaction containing 20 mMTris-HCI (pH 7.4), 5 mM $MgCl_2$, 50 mM KCI and 80 units of *Spe*I (Life Technologies, Gaithersburg, MD, USA). The nucleotide sequence of the linearized plasmid is shown in Figures 2 (SEQ ID No.9).

IFN-γ RNA was generated from 2 μg of the linearized plasmid in a 50 μL *in vitro* transcription reaction using the following materials:

40 mM Tris-HCI (pH 8.0)

8 mM $MgCl_2$

2 mM spemidine - $(HCl)_3$

20 mM DTT (dithiothreotol)

1 mM ATP (adenosine triphosphate)

1 mM GTP (guanosine triphosphate)

1 mM CTP (cytidine triphosphate)

1 mM UTP (uridine triphosphate)

100 units of RNasin® RNase inhibitor (Promega, Madison, WI, USA)

250 units of T7 RNA polymerase (Life Technologies, Gaithersburg, MD, USA).

The reaction was incubated at 37°C for 60 minutes. To digest the DNA template, 5 units of *DNase*I was added

to the reaction which was then incubated a further 15 mintues at 37°C. The IFN-γ RNA standard was then purified away from the salt, enzymes, unincorporated NTP's and digested DNA fragments by passing the reaction mix through a CHROMA SPIN™-30 DEPC-H_2O columns (Clonetech, Palo Alto, CA, USA) according to the manufacturer's instructions.

The IFN-γ RNA standard was then quantified by measuring its OD at a wavelength of 260 nm (1 OD at 260 nm = 40 µg of RNA). Since the RNA has a length of 738 bases and each base has a molecular weight (MW) of 340 daltons, the MW of this RNA standard can be calculated (738 X 340 - 250,920 daltons). If the mass of the RNA is divided by its MW, the number of moles (e.g. 1 µg of the RNA standard contains 1 X 10^{-6} g/250,920 daltons = 3.9853 X 10^{-12} moles or 3.9853 pmols) can be determined. Since 1 mole of any compound contains 6.022 X 10^{23} molecules, the number of copies of RNA that are in 1 µg of the IFN-γ RNA standard can be determined (1 µg contains 3.9853 X 10^{-12} moles X 6.02 X 10^{23} = 2.3999 X 10^{12} copies of the RNA standard per 1 µg). The standard can then be diluted to give any copy number desired in each reaction. The nucleotide sequence of the IFN-γ RNA standard transcript is shown in Figure 3 (SEQ ID No. 10).

Log dilutions of the RNA standard were used to prepare a curve of threshold values (Ct) plotted against log input copy number and is shown in Figure 4.

SUMMARY OF THE DISCLOSURE

In summary of this disclosure, the present invention provides an accurate method of determining the quantity of specific RNA in cells, to permit

analysis of rare transcripts, such as cytokines, based on a modified RNA isolation procedure, RT-PCR in a single enzyme reaction, detection and comparison to a RNA standard. Modifications are possible within the scope of this invention.

TABLE I

Sample	Immunization	Copy #	+/-SD
529	PBS	305.82	66.67
549	Live RSV	15704.12	1575.72
569	FI RSV	7328.56	799.68
509	Live RSV	15399.17	818.21
550	PBS	1873.54	328.59
551	PBS	2732.02	346.20

FI - Formalin inactivated RSV

PBS - Phosphate buffered saline

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